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(54) Title: ANTIBODY PURIFICATION

(57) Abstract

This invention relates to the application of hydrophobic interaction chromatography combination chromatography to the purificatio of antibody molecule proteins. The process may include the sequential steps of Protein A affinity chromatography, ion exchan chromatography, and hydrophobic interaction chromatography. Purified monomeric IgG antibody, free of immunoglobulin aggregate misfolded species, host cell protein, and Protein A, may be recovered by the process.

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Antibody Purification

Field of the Invention:

This invention relates to the field of protein purification. More specifically, this invention relates to the application of Hydrophobic Interaction Chromatography (HIC) to the separation of Immunoglobulin G monomers and to the integration of HIC into a combination chromatographic protocol for the purification of IgG antibody molecules.

Background of the Invention:

Historically, protein purification schemes have been predicated on differences in the molecular properties of size, charge and solubility between the protein to be purified and undesired protein contaminants. Protocols based on these parameters include size exclusion chromatography, ion exchange chromatography, differential precipitation and the like.

Size exclusion chromatography, otherwise known as gel filtration or gel permeation chromatography, relies on the penetration of macromolecules in a mobile phase into the pores of stationary phase particles. Differential penetration is a function of the hydrodynamic volume of the particles. Accordingly, under ideal conditions the larger molecules are excluded from the interior of the particles while the smaller molecules are accessible to this volume and the order of elution can be predicted by the size of the protein because a linear relationship exists between elution volume and the log of the molecular weight.

Chromatographic supports based on cross-linked dextrans e.g. SEPHADEX®, spherical agarose beads e.g. SEPHAROSE® (both commercially available from Pharmacia AB. Uppsala, Sweden), based on cross-linked polyacrylamides e.g. BIO-GEL® (commercially available from BioRad Laboratories, Richmond, California) or based on ethylene glycol-methacrylate copolymer e.g. TOYOPEARL HW65 (commercially available from Toso Haas Co., Tokyo, Japan) are useful in forming the various chromatographic columns for size exclusion, or HIC chromatography in the practice of certain aspects of this invention.

Precipitation methods are predicated on the fact that in crude mixtures of proteins the solubilities of individual proteins are likely to vary widely. Although the solubility of a protein in an aqueous medium depends on a variety of factors, for purposes of this discussion it can be said generally that a protein

(See: Ey, P.L. et al. Immunochemistry 15:429-36 (1978)). Alternatively, antisera raised in heterologous species (e.g. rabbit anti-mouse antisera) can be used to separate general groups of antibodies. (See, Current Protocols in Molecular Biology Supra, Chap 11.)

Hydrophobic interaction chromatography was first developed following the observation that proteins could be retained on affinity gels which comprised hydrocarbon spacer arms but lacked the affinity ligand. Although in this field the term hydrophobic chromatography is sometimes used, the term hydrophobic interaction chromatography (HIC) is preferred because it is the interaction between the solute and the gel that is hydrophobic not the chromatographic procedure. Hydrophobic interactions are strongest at high ionic strength, therefore, this form of separation is conveniently performed following salt precipitations or ion exchange procedures. Elution from HIC supports can be effected by alterations in solvent, pH, ionic strength, or by the addition of chaotropic agents or organic modifiers, such as ethylene or propylene glycol. A description of the general principles of hydrophobic interaction chromatography can be found in U. S. Patent 3,917,527 and in U. S. Patent 4,000,098. The application of HIC to the purification of specific proteins is exemplified by reference to the following disclosures: human growth hormone (U. S. Patent 4,332,717), toxin conjugates (U. S. Patent 4,771,128), antihemolytic factor (U. S. Patent 4,743,680), tumor necrosis factor (U. S. Patent 4,894,439), interleukin-2 (U. S. Patent 4,908,434), human lymphotoxin (U. S. Patent 4,920,196) and lysozyme species (Fausnaugh, J.L. and F. E. Regnier, J. Chromatog. 359:131-146 (1986)) and soluble complement receptors (U.S. Patent 5,252,216). HIC in the context of high performance liquid chromatography (HPLC) has been used to separate antibody fragmens (e.g., $F(ab')_2$) from intact antibody molecules in a single step protocol. (Morimoto, K. et al., J. Biochem. Biophys. Meth. 24: 107-117 (1992)).

In addition to affinity and HIC techniques, one or more of the traditional protein purification schemes have been applied to antibody purification. For example, Hakalahti, L. et al., (J. Immunol. Meth. 117: 131-136 (1989)) disclose a protocol employing two successive ion exchange chromatographic steps or one employing a single ion exchange step followed by a HIC step. Danielsson A. et al. (J. Immunol. Methods 115: 79-88 (1988)) compare single step protocols based on anion exchange, cation exchange, chromatofocusing and HIC respectively.

IgG of >95% protein purity. The invention may be applied to the purification of a number of different immunoglobulin G molecules.

Antibody-like proteins are proteins which may be purified by the protocol described herein, such protocol being modified if necessary by routine, non-inventive adjustments that do not entail undue experimentation. Such proteins include isotypes, allotypes and alleles of immunoglobulin genes, truncated forms, altered antibodies, such as chimeric antibodies, humanized antibodies and the like, chemically modified forms such as by PEG treatment, and fusion proteins containing an immunoglobulin moiety. These proteins are referred to as antibody-like because they possess or retain sufficient immunoglobulin protein properties (e.g. F_c determinants) to admit to purification by the process of this invention. Unless specifically identified otherwise, the term antibody or immunoglobulin protein also includes antibody-like proteins.

The immunoglobulin molecules of this invention can be isolated from a number of sources, including without limitation, serum of immunized animals, ascites fluid, hybridoma or myeloma supernatants, conditioned media derived from culturing a recombinant cell line that expresses the immunoglobulin molecule and from all cell extracts of immunoglobulin producing cells. This invention is particularly useful for the purification of antibodies from conditioned cell culture media of a variety of antibody producing recombinant cell lines. Although one may expect some variation from cell line to cell line and among the various antibody products, based on the disclosure herein, it is well within the purview of one of ordinary skill in this art to adapt the invention herein to a particular combination of antibody protein and producing cell line.

Generally, genes encoding proteins such as antibodies may be cloned by incorporating DNA sequences coding for the desired regions of the polypeptide into a recombinant DNA vehicle (e.g., vector) and transforming or transfecting suitable prokaryotic or eukaryotic hosts. Suitable prokaryotic hosts include but are not limited to Escherichia, Streptomyces, Bacillus and the like. Suitable eukaryotic hosts include but are not limited to yeast, such as Saccharomyces and animal cells in culture such as VERO, HeLa, mouse C127, Chinese hamster ovary (CHO), WI-38, BHK, COS, MDCK, myeloma, and insect cell lines. Particularly preferred hosts are CHO cell lines deficient in dihydrofolate reductase such as ATCC CRL 1793, CRL 9096 and other cell lines described herein below. Such recombinant techniques have now become well known and are described in Methods in Enzymology, (Academic Press) Volumes 65 and 69

possible to assemble fragments of a single coding sequence such that upon expression an antibody molecule is formed. A particularly efficacious application of this protocol to recombinant antibody production is found in the Harris, et al. PCT Applications WO92/04381, published March 19, 1992, cited above, and in the Newman et al. PCT Application WO93/02108, published February 4, 1993, cited above.

After the recombinant product is produced it is desirable to recover the product. If the product is exported by the cell producing it, the product can be recovered directly from the cell culture medium. If the product is retained intracellularly, the cells must be physically disrupted by mechanical, chemical or biological means in order to obtain the intracellular product.

In the case of a protein product, the purification protocol should not only provide a protein product that is essentially free of other proteins, by which is meant at least 80% and preferably greater than 95% pure with respect to total protein in the preparation, but also eliminate or reduce to acceptable levels other host cell contaminants, DNA, RNA, potential pyrogens and the like. Furthermore, in the context of antibody production by recombinant expression system, it is appreciated that aggregation of the 150,000 dalton IgG product into higher molecular weight species can occur. Accordingly, for purposes of product purity and standardization it is also useful to separate the native 150,000 dalton monomeric species from higher molecular weight aggregates and other misfolded forms. While it is appreciated that the 150,000 dalton IgG species is composed of four polypeptide chains (2 heavy chains and 2 light chains), the 150,000 dalton species is referred to herein as a "monomer" or "monomeric IgG".

As mentioned above, a variety of host cells may be used for the production of the antibodies of this invention. The choice of a particular host cell is well within the purview of the ordinary skilled artisan taking into account, inter alia, the nature of the antibody, its rate of synthesis, its rate of decay and the characteristics of the recombinant vector directing the expression of the antibody. The choice of the host cell expression system dictates to a large extent the nature of the cell culture procedures to be employed. The selection of a particular mode of production, be it batch or continuous, spinner or air lift, liquid or immobilized can be made once the expression system has been selected. Accordingly, fluidized bed bioreactors, hollow fiber bioreactors, roller bottle cultures, or stirred tank bioreactors, with or without cell microcarriers may variously be employed. The criteria for such selection are appreciated in

density of the commercially available phenyl or octyl phenyl gels is on the order of 40 μmoles/ml gel bed. Gel capacity is a function of the particular protein in question as well as pH, temperature and salt type and concentration but generally can be expected to fall in the range of 3-20 mg/ml of gel.

The choice of a particular gel can be determined by the skilled artisan. In general the strength of the interaction of the protein and the HIC ligand increases with the chain length of the alkyl ligands but ligands having from about 4 to about 8 carbon atoms are suitable for most separations. A phenyl group has about the same hydrophobicity as a pentyl group, although the selectivity can be quite different owing to the possibility of pi-pi orbital interaction with aromatic groups on the protein. Selectively may also be affected by the chemistry of the supporting resin.

Adsorption of the proteins to a HIC column is favored by high salt concentrations, but the actual concentrations can vary over a wide range depending on the nature of the protein and the particular HIC ligand chosen. Various ions can be arranged in a so-called soluphobic series depending on whether they promote hydrophobic interactions (salting-out effects) or disrupt the structure of water (chaotropic effect) and lead to the weakening of the hydrophobic interaction. Cations are ranked in terms of increasing salting out effect as $Ba^{++} < Ca^{++} < Mg^{++} < Li^{+} < Cs^{+} < Na^{+} < K^{+} < Rb^{+} < NH_4^{+}$, while anions may be ranked in terms of increasing chaotropic effect as $PO_4^{---} < SO_4^{--} < CH_3COO^{-} < Cl^{-} < Br^{-} < NO_3^{-} < ClO_4^{-} < I^{-} < SCN^{-}$.
Accordingly, salts may be formulated that influence the strength of the interaction as given by the following relationship:

$$(NH_4)_2SO_4 > Na_2SO_4 > NaCl > NH_4Cl > NaBr > NaSCN$$

In general, salt concentrations of between about 0.75 and about 2M ammonium sulfate or between about 1 and 4M NaCl are useful.

The influence of temperature on HIC separations is not simple, although generally a decrease in temperature decreases the interaction. However, any benefit that would accrue by increasing the temperature must also be weighed against adverse effects such an increase may have on the stability of the protein.

Elution, whether stepwise or in the form of a gradient, can be accomplished in a variety of ways: (a) by changing the salt concentration, (b) by changing the polarity of the solvent or (c) by adding detergents. By decreasing salt concentration adsorbed proteins are eluted in order of increasing hydrophobicity. Changes in polarity may be affected by additions of solvents such as ethylene or propylene glycol or (iso)propanol, thereby decreasing the

cross-linked ion exchangers are also known. For example, DEAE-, QAE-, CM-, and SP- SEPHADEX® and DEAE-, Q-, CM-and S-SEPHAROSE® and SEPHAROSE® Fast Flow are all available from Pharmacia AB. Further, both DEAE and CM derivitized ethylene glycol-methacrylate copolymer such as TOYOPEARL DEAE-650S or M and TOYOPEARL CM-650S or M are available from Toso Haas Co., Philadelphia, Pa. Because elution from ion exchange supports usually involves addition of salt and because, as mentioned previously, HIC is enhanced under increased salt concentrations, the introduction of a HIC step following an ionic exchange chromatographic step or other salt mediated purification step is particularly preferred. Additional purification protocols may be added including but not necessarily limited to further ionic exchange chromatography, size exclusion chromatography, viral inactivation, concentration and freeze drying.

For purposes of illustration only, this invention was applied to the purification of several antibodies of the IgG isotype. More specifically, to a humanized antibody useful for the treatment of RSV infection described by Harris et al.; 1992, Intl. Patent Publication Number WO92/04381, published March 19, 1992 (hereinafter "RSHZ-19") and a chimeric antibody specifically reactive with the CD4 antigen described by Newman et al. Int'l Patent Publication Number WO93/02108, published February 4, 1993 (hereinafter CH-CD4). The construction of recombinant systems for the production of RSHZ-19 and the CH-CD4 chimeric antibodies are detailed in the above mentioned PCT Applications, the contents of which are incorporated herein by reference for purpose of background and are summarized as follows.

An expression plasmid containing the RSHZ-19 coding sequence was co-transfected with pSV2dhfr into a dhfr-requiring Chinese Hamster Ovary cell line (CHODUXBII). The transfection was carried in growth medium and employed the calcium coprecipitation/ glycerol shock procedure as described in: DNA Cloning, D.M. Glover ed. (Chap. 15, C. Gorman). Following transfection, the cells were maintained in growth medium for 46 hours under growth conditions (as described above) prior to the selection procedure.

The selection and co-amplification procedure was carried out essentially as described by R.J. Kaufman, et al.(Mol. Cell. Biol. 5:1750-1759 (1985)). Forty-six hours post transfection the cells were changed to selective medium MEM ALPHA (041-02571), 1% stock glutamine, 1% stock pen/strep (043-05070) and dialyzed bovine fetal calf serum (220-6300AJ) (Gibco, Paisley, Scotland). The cells were maintained in the selective medium for 8-10 days

EXAMPLE 1

INTRODUCTION

The procedure outlined below was developed for the isolation and purification of a monoclonal antibody against Respiratory Syncytial Virus (RSV). This antibody is a "humanized" IgG expressed in CHO cells, and grown in a stirred tank bioreactor. The antibody is more fully described in PCT WO92/04381 and is otherwise referred to herein as RSHZ 19. The process is designed to prepare RSHZ-19 of >95% purity while removing contaminants derived from the host cell, cell culture medium, or other raw materials. The process in its most preferred embodiment consists of three purification steps (Protein A affinity, cation exchange, and hydrophobic interaction chromatography), two viral inactivation steps, and a diafiltration step to exchange the product into a final buffer of choice (outlined in Figure 1). All steps are carried out at room temperature (18 - 25 °C). All buffers are prepared with WFI and filtered through either a 0.2 micron filter or a 10,000 MWCO membrane before use. Buffer formulations are listed in Table 1. Tables 2, 4, 6 and 8 show the column parameters for examples IA, IB, IC and ID respectively. Tables 3, 5 and 7 and 9 provide a purification summary for examples IA, IB, IC and ID respectively.

The first step in the process (Protein A affinity chromatography on ProSep A) can be rapidly cycled to accommodate varying amounts of cell-free culture fluid (CCF), and has a capacity of approximately 15 grams RSHZ-19 per liter of ProSep A. For example, 500 liters CCF containing 400 - 500 grams of IgG can be processed in 5 or 6 cycles. The downstream steps of the process (Cation Exchange Chromatography (CEC) and Hydrophobic Interaction Chromatography (HIC) are scaled to accommodate approximately 130 - 140 grams RSHZ-19 per cycle. Thus, a 500 liter culture containing 400 - 500 grams of RSHZ-19 is processed in three downstream cycles after capture on ProSep A.

The hydrophobic interaction chromatography step (HIC) has been demonstrated to remove residual Protein A that leaches from the Protein A column during elution (See: examples IA-D). In addition, aggregates of IgG can be removed over HIC, as shown in examples IC and ID.

0.2 µm filter (Millipore Millipak or equivalent), and held in sterile containers at 4 °C, or frozen and held at -70 °C.

The pH 3.5 treatment provides viral inactivation, and the pH 5.5 adjustment prepares the solution for cation exchange chromatography (CEC). The pH 3.5 treatment can be omitted if desired.

Cation Exchange Chromatography

The pH inactivated Protein A eluate is further purified by CEC chromatography on column of CM SEPHAROSE FF (Pharmacia LKB). The sample is applied to the equilibrated column at a flow rate of 150 cm/hr and a load ratio of ≤20 grams protein per liter CM SEPHAROSE. After loading, the column is washed with 3 to 5 column volumes of Equilibration Buffer. The product is eluted with 3 - 5 column volumes of Elution Buffer.

The cation exchange chromatography step removes protein and non-protein impurities.

Viral Inactivation with Guanidine

The cation exchange eluate is adjusted to approximately 2.0 M guanidine hydrochloride by the slow addition (with mixing) of one-half volume of Guanidine Stock Solution. The rate of reagent addition is adjusted so that it is added over a 5 - 15 minute period. The solution is transferred to a second vessel, and is held for thirty minutes to achieve viral inactivation. After holding, an equal volume of Ammonium Sulfate Stock Solution is slowly added (with mixing), and the hydrophobic interaction chromatography (HIC) step is performed immediately. The rate of reagent addition is adjusted so that it is added over a 5 - 15 minute period.

The guanidine treatment provides a second viral inactivation step, when an acid inactivation step is employed, and keeps the RSHZ-19 soluble after ammonium sulfate addition; the addition of ammonium sulfate serves to dilute the guanidine and prepare the solution for HIC.

Pooling Criteria

The eluate fractions from the Protein A capture and cation exchange steps are pooled based on the UV tracing on the chromatogram, and the entire peak is collected. The eluate from the HIC step is pooled based on the UV tracing, and the main peak is pooled until the UV reading on the tailing side of the peak reaches 20% of the peak maximum. The HIC tail fraction contains the majority of the Protein A and aggregated IgG's.

Example IA. RSHZ-19 purification at 1 gram scale using TOYOPEARL Phenyl-650M

A 5.0 liter (20 cm diameter by 16 cm length) ProSep A affinity column was equilibrated with PBS (see Table 1) at 5.2 liter/min. 100 liters of conditioned culture medium containing 0.8 grams per liter of RSHZ-19 monoclonal antibody was clarified by microfiltration as described above, and applied to the column at a flow rate of 5.2 liter/min. After the load, approximately 15 liters of PBS/glycine was applied to the column at the same flow rate. The IgG was eluted by applying 15 - 20 liters of ProSep A elution buffer. Fractions of the non-bound peak and the elution peak were collected and assayed for IgG content using an HPLC assay. The eluate was approximately 15 liters in volume, and contained approximately 5 milligrams protein per milliliter.

Immediately after elution, the sample was adjusted to pH 3.5 by the addition of 2.5 M hydrochloric acid, held for approximately 30 minutes, and adjusted to pH 5.5 by the addition of approximately 350 milliliters of 1 M Tris base. After neutralizing to pH 5.5, the sample was filtered through a 0.1 micron Polygard CR filter in tandem with a sterile 0.2 micron Millipak 200, into a sterile container. The filtrate was stored at 4 °C. Samples of the filtrate were analyzed for IgG content using an HPLC assay, and for total protein by absorbance at 280 nanometers. The samples were also analyzed for Protein A content by an ELISA procedure. This pH 3.5 treated and filtered Prosep A eluate was used as the CM SEPHAROSE load in Examples IA, B and C.

400 milliliters of pH 3.5 treated and filtered ProSep A eluate were loaded directly onto a 220 milliliter (4.4 cm diameter x 15 cm length) column of CM SEPHAROSE FF at 38 mL/min, which had been previously equilibrated with CM Equilibration buffer. After loading, the column was washed at 38 mL/min with approximately 700 milliliters of CM Equilibration Buffer. The IgG was eluted by applying CM Elution Buffer at 38 mL/min. The IgG came off the column after approximately 1 bed volume of Elution Buffer had passed. The entire peak was collected as CM SEPHAROSE eluate. Fractions of the CM non-bound, eluate and strip fractions were collected and analyzed for IgG content, total protein content, and Protein A content as described previously. The eluate was approximately 160 milliliters in volume, and contained approximately 12 milligrams protein per milliliter. This CM

Table 2: Column Parameters at 1 gram scale using Phenyl-650M

Step	Column Volume (liter)	Column dia x length (cm)	Load Ratio	Flow Rates (cm/hr)	Flow Rates (mL/min)
ProSep A	5.0	20 x 16	16.0 g IgG per liter bed volume	1000	5200
CM SEPHAROSE FF	0.22	4.4 x 15	9.1 g protein per liter bed volume	150	38
Phenyl-650M	0.08	3.2 x 10	10.4 g protein per liter bed volume	150	20

Example IB. RSHZ-19 purification at 1 gram scale using TOYOPEARL Butyl-650M

This preparation used the same CM SEPHAROSE eluate as described in Example IA, and the HIC step was performed using TOYOPEARL Butyl-650M instead of Phenyl-650M. The preparation of the CM SEPHAROSE eluate is described in Example IA above. To 80 milliliters of CM SEPHAROSE eluate was added (slowly with constant stirring) a total of 40 milliliters of Guanidine Stock Solution. This brought the guanidine concentration to 2 M for viral inactivation. While stirring the guanidine-treated solution, a total of 120 milliliters of 2.0 M Ammonium Sulfate Stock Solution was added. The resulting solution was 1.0 M in guanidine and 1.0 M in ammonium sulfate. The ammonium sulfate treated solution was applied to a an 80 mL column (3.2 cm diameter x 10 cm length) of TOYOPEARL Butyl-650M, previously equilibrated with Butyl Equilibration Buffer. The flow rate was 20 mL/min throughout the run. After loading, the column was washed with approximately 350 milliliters of Butyl Equilibration Buffer. The IgG was eluted by applying a linear gradient starting at 65% Equilibration/35% Gradient buffer and ending at 20% Equilibration/80% Gradient buffer, in 12 - 13 column volumes. This represents a starting ammonium sulfate concentration of approximately 0.65 M and an ending concentration of approximately 0.2 M. The slope of this gradient was approximately a 3.3% increase in elution buffer per column volume, or -0.033 M ammonium sulfate per column volume. The IgG began to elute from the column at approximately 2 column volumes and ended at approximately 9 column volumes into the gradient (ammonium sulfate concentration of approximately 0.58 to 0.35 M). The eluate fraction was collected until the UV absorbance on the tailing side of the peak decreased to 10% of the peak height, then collection was switched to another vessel. At the end of the gradient, approximately 250 mL of Butyl Gradient Buffer was applied, and a small peak eluted and was collected. Approximately 250 mL of HIC Strip Buffer was applied to regenerate the column. Fractions of the Butyl non-bound, eluate, tail and strip fractions were collected and analyzed for IgG content, total protein content, and Protein A content as described previously. The eluate was approximately 400 milliliters in volume, and contained approximately 1.5 milligrams protein per milliter.

Table 4 summarizes the column parameters for this example. The product and protein recovery data for each step are shown in Table 5, along with the Protein A content, expressed as nanograms Protein A per milligram IgG (ng/mg). Although

Table 5: Purification Summary for Example IB, 1 gram scale using Butyl-650M

Step	Volume (Liters)	Total RSHZ-19[a] (Grams)	Total Protein[b] (Grams)	Step Yield (%)	Protein A[c] (ng/mg)
Cell-free Culture Fluid	100	80.3	n.d.	--	0
ProSep A Eluate	15.8	73.8	80.4	92	20.2
CM SEPHAROSE[d] Load	(0.4)[d]	(1.87)[d]	(2.04)[d]		
CM SEPHAROSE Eluate	0.16	2.01	1.88	100	14.5
Butyl-650M[d] Load	(0.21)[d]	(0.76)[d]	(0.86)[d]		
Butyl-650M Eluate	0.41	0.60	0.62	79	0.7
(Butyl Tail	0.40	0.03	0.03	--	31.4)[e,f]
(Butyl Strip	0.53	0.10	0.10	--	n.d.)[g]
Cumulative Recovery (%)				73	
Mass Balance (% of load)				95	

[a] by HPLC
[b] by Absorbance at 280 nm ÷ 1.27 mL mg^{-1} cm^{-1}
[c] by ELISA
[d] Only a portion of the total eluate from the previous column was carried forward, as described above
[e] Protein A migrates primarily in the Tail fraction
[f] Tail contains 3% of the load
[g] strip contains 13% of the protein that was loaded

column volumes into the gradient (ammonium sulfate concentration of approximately 0.85 to 0.5 M). The eluate fraction was collected until the UV absorbance on the tailing side of the peak decreased to 20% of the peak height, then collection was switched to another vessel (tail). Fractions of the Phenyl non-bound, eluate and tail and strip fractions were collected and analyzed for IgG content, total protein content, and Protein A content as described previously. The eluate was approximately 15.4 L in volume, and contained approximately 2.2 milligrams protein per milliter.

The Phenyl Eluate was concentrated to approximately 16 mg/mL using a tangential flow ultrafiltration apparatus (CUF, Millipore Corp.) equipped with 30,000 MWCO Omega membranes (Filtron Corp.) and buffer exchanged by continuous diafiltration against a suitable formulation buffer.

Table 6 summarizes the column parameters for this example. The product and protein recovery data for each step are shown in Table 7, along with the Protein A content, expressed as nanograms Protein A per milligram IgG (ng/mg) and the IgG aggregate content, expressed as % of total IgG. As seen in Table 7, the Protein A reduction over Phenyl-650M is approximately 3-fold, and the recovery is approximately 90%. IgG aggregates were reduced from 0.5% in the CM SEPHAROSE eluate to 0.06% in the formulated product.

Table 6: Column Parameters at 40 gram scale

Step	Column Volume (liter)	Column dia x length (cm)	Load Ratio	Flow Rates (cm/hr)	(L/min)
CM SEPHAROSE FF	4.2	25 x 8.5	8.9 g protein per liter bed volume	150	1.2
Phenyl-650M	4.6	18 x 18	10.4 g protein per liter bed volume	140	0.6

Example ID. RSHZ-19 purification at 125 gram scale using TOYOPEARL Phenyl-650M

A 5.5 liter (20 cm diameter by 18 cm length) ProSep A affinity column was equilibrated with PBS (see Table 1) at 4.8 liter/min. 450 liters of conditioned culture medium containing 0.94 grams per liter of RSHZ-19 monoclonal antibody was clarified by microfiltration as described above, and applied in four separate 90 - 95 liter portions and one 40 liter portion to the column at a flow rate of 4.8 liter/min (and so throughout). Each cycle on the column ran as follows: After the load, approximately 17 liters of PBS/glycine was applied to the column at the same flow rate. The IgG was eluted by applying 15 - 20 liters of ProSep A Elution buffer. Fractions of the non-bound peak and the elution peak were collected and assayed for IgG content using an HPLC assay. The eluate from each cycle was approximately 9 liters in volume, and contained approximately 5 - 10 milligrams protein per milliliter. Immediately after elution, the ProSep A eluates were adjusted to pH 3.5 by the addition of 2.5 M hydrochloric acid, held for approximately 30 minutes, and adjusted to pH 5.5 by the addition of approximately 250 milliliters of 1 M Tris base. After neutralizing to pH 5.5, the eluates were pooled together, and filtered through a 0.1 micron Polygard CR filter in tandem with a sterile 0.2 micron Millipak 200, into 5 liter aliquots in sterile containers. The filtrate was stored at 4 °C. Samples of the filtrate were analyzed for IgG content using an HPLC assay, and for total protein by absorbance at 280 nanometers. The samples were also analyzed for Protein A content by an ELISA procedure, and IgG aggregates by HPLC.

The downstream steps were scaled-up to accommodate approximately 120 - 140 grams of protein. 16.3 liters of pH 3.5 treated and filtered ProSep A eluate containing approximately 130 grams of protein was loaded directly onto a 14.4 liter (35 cm diameter x 15 cm length) column of CM SEPHAROSE FF at 2.4 L/min, which had been previously equilibrated with CM Equilibration buffer. After loading, the column was washed at 2.4 L/min with approximately 45 liters of CM Equilibration Buffer. The IgG was eluted by applying CM Elution Buffer at 2.4 L/min. The IgG began to elute from the column after approximately 1 - 2 bed volumes of Elution Buffer had passed. The entire peak was collected as CM SEPHAROSE eluate. Fractions of the CM non-bound and eluate were collected and analyzed for IgG content, total protein content, and IgG aggregate. The eluate was approximately 21 liters in volume, and contained approximately 120 grams protein.

the cumulative recovery is approximately 70%. IgG aggregates were reduced from 0.4% in the CM SEPHAROSE eluate to 0.06% in the formulated product.

Table 8: Column Parameters at 125 gram scale

Step	Column Volume (liter)	Column dia x length (cm)	Load Ratio	Flow Rates (cm/hr)	(L/min)
ProSep A	5.0	20 x 18	14 - 16 g RSHZ-19 per liter bed volume	915	4.8
CM SEPHAROSE FF	14.4	35 x 15	8.4 g protein per liter bed volume	150	2.4
Phenyl-650M	12.4	30 x 18	8.6 g protein per liter bed volume	100	1.2

Table 10: Purity analysis: 125 Gram Scale

Step	Aggregates (% of Total IgG)	Purity[a] (% of Total Area)	Activity[b] (%)
CCF	not applicable	not done	80[c]
ProSep Eluate	0.4	98.5	105
CM Eluate	0.4	98.4	109
Phenyl Eluate	<0.05	98.3	99
Final product	0.06	99.7	115

[a] Determined by scanning densitometry of reducing SDS-PAGE; sum of area of Heavy and Light chains of IgG

[b] Calculated ratio of activity (determined by Bovine RS Virus binding ELISA) to RSHZ-19 concentration (determined by A280)

[c] Calculated ratio of activity, by bovine RS virus ELISA, to RSHZ-19 concentration by HPLC

TABLE 11

Fraction No.	Volume (mL)	Product (mg/mL)	Protein A (ng/mL)	Cumulative Specific Protein A (ng/mg)	Cumulative Product Yield	Protein A Removal Factor[a]
	Load					
0	12	5.8	200.7	35		
	Eluate					
1	5.5	0.04	0.0	0	0%	
2	3	0.31	0.0	0	2%	
3	4.2	1.32	6.4	4	10%	8.6
4	4.2	3.1	16.9	5	28%	7.0
5	4	4	68.3	10	51%	3.3
6	4.2	3.4	80.9	14	72%	2.4
7	4.2	2.3	94.1	19	86%	1.9
8	4.2	1.3	79.3	22	94%	1.6
9	4.2	0.71	49.9	24	98%	1.4
10	4.2	0.36	32.8	26	100%	1.3
11	4	0.19	22.9	27	101%	1.3
12	4	0.11	13.5	27	102%	1.3
13	9.6	0.05	8.5	28	102%	1.2

[a] Removal factor is calculated by pooling eluate fractions from start of eluate to desired fraction and dividing initial Protein A in load by Protein A ng/mg in pooled eluate fractions. For example, the factor 8.6 is calculated by dividing the sum of Protein A in fractions 1-3 by the sum of product in fractions 1-3 to get 4 ng/mg. This number is then divided by 35 ng/mg in the load to obtain 8.6.

Comparing the results of example IIB with example IIA it can be seen that Protein A was reduced by 6 to 8 fold with about 80% yield when the pH 3.5 wash was included, but reduction was only about 2 fold with 80% yield of CH-CDH without the pH 3.5 wash.

EXAMPLE IIC:

This example was performed similar to example IIB, except that the scale was increased. Equilibration and Wash buffers are described in example IIB. The column was 5 cm in diameter and 28 cm high and the flowrate was 50 mL/min. CH-CD4 monoclonal antibody was prepared and partially purified, as described in example IIA. The partially purified product (440 mL) was mixed with 220 mL 6 M guanidine HCl for 31 min. Then, 660 mL 2 M ammonium sulfate, 50 mM sodium phosphate , pH 7.0 was added. The final ammonium sulfate concentration was 1 M and the final antibody concentration was 5.3 mg/mL. After sampling, the load volume was 1,290 mL.

The column was equilibrated with 2 column volumes of Equilibration buffer and then loaded on the column. The column was then washed with 630 mL of Equilibration buffer, 1,000 mL of Wash buffer, 800 mL of Equilibration buffer, and then eluted with a 5 column volume gradient from 0.75 M ammonium sulfate, 50 mM sodium phosphate, pH 7.0, to 50 mM sodium phosphate, pH 7.0. Fractions were collected during elution.

The results are presented in Table 13. Protein A was reduced 100 fold with a yield of 70%, or by 30 fold with an antibody yield of 80%.

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EXAMPLE IID

Partially purified CH-CD4 monoclonal antibody was prepared as shown in example IIA. Equilibration and Wash buffers are described in example IIB. Four milliliters of 6 M guanidine HCl, 50 mM sodium phosphate, pH 7.0 was added to 8 mL of a 9.5 mg/mL solution of partially purified CH-CD4 monoclonal antibody and incubated for 30 minutes. Then, 12 mL of 2 M ammonium sulfate, 50 mM sodium phosphate, pH 7.0 was added slowly. The column was 0.5 cm in diameter and 20 cm high (4 mL) and the flowrate for all steps was 0.5 mL/min. The column was rinsed with 2 column volumes each of water and Equilibration buffer. Then, 22 mL of the load solution was passed through the column, followed by 2 column volumes of Equilibration buffer, followed by Wash buffer until the pH of the column effluent was 3.5. This was followed by Equilibration buffer until the effluent pH was 7.0. The column was eluted with 0.3 M ammonium sulfate, 50 mM sodium phosphate, pH 7.0. After the UV trace started to rise, 12.6 mL of eluate were collected and analyzed for product and Protein A. The yield of product was 80% and the Protein A was reduced from 28 ng/mg to 6 ng/mg, a reduction of 4.7 fold.

13. The method according to Claim 9 wherein the hydrophobic interaction chromatographic employs a support selected from the group consisting of alkyl C_2-C_8-agarose, aryl-agarose, alkyl-silica, aryl-silica, alkyl-organic polymer resin and aryl-organic polymer resin.

14. The method according to Claim 13 wherein the support is selected from the group consisting of butyl-, phenyl- and octyl-agarose and butyl-, phenyl- and ether-organic polymer resin.

15. The method according to Claim 14 wherein the support is phenyl-organic polymer resin or butyl-organic polymer resin.

16. The method according to Claim 9 wherein the support is phenyl- or butyl-organic polymer resin and the antibody is selectively eluted with a low salt buffer.

17. The method according to Claim 16 wherein the antibody is selectively eluted with a gradient decreasing to 50 mM sodium phosphate buffer, pH 7.0.

18. The method according to Claim 9 wherein the Protein A chromatography employs as a support Protein A linked to controlled pore glass and elution is by a low pH buffer.

19. The method according to Claim 18 wherein said buffer is 25 mM citrate, pH 3.5.

20. A method for purifying antibody from a conditioned cell medium comprising:

(a) adsorbing the antibody onto a Protein A chromatographic support;

(b) washing the adsorbed antibody with at least one buffer;

(c) eluting the antibody from step (b);

(d) adsorbing the antibody from step (c) onto an ion exchange chromatographic support;

(e) washing the absorbed antibody with at least one buffer;

(f) selectively eluting the antibody from step (e);

(g) adsorbing the eluate of step (f) onto a hydrophobic interaction chromatographic support;

(h) washing the adsorbed antibody with at least one buffer;

(i) eluting the adsorbed antibody; and

(j) recovering the antibody.

21. The method according to Claim 20 which includes one or more optional steps of inactivating viruses if present.

35. The method according to Claim 20 wherein the absorbed antibody of step (h) is washed with two buffers, a first equibration buffer and a second low pH wash buffer.

36. The method according to Claim 35 wherein the pH of the second buffer is less than 4.0.

37. The method according to Claim 36 wherein the second buffer is 1 M ammonium sulfate, 50 mM sodium citrate, pH 3.5.

38. A method for removing Protein A from a mixture comprising Protein A and antibodies comprising contacting said mixture with a hydrophobic interaction chromatography support and selectively eluting the antibody from the support.

39. The method according to Claim 38 which includes washing the support prior to elution with a buffer having a pH less than 7.0.

40. The method according to Claim 39 wherein the pH of the wash buffer is less than 4.0.

41. The method according to Claim 40 wherein the buffer is (1 M ammonium sulfate, 50 mM sodium citrate, pH 3.5).

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/01823

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :B01D 15/08
US CL : 210/635, 656; 530/390.5, 413, 417
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 210/635, 656, 198.2; 530/387.1, 390.5, 413, 417

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,164,487 (KOTHE ET AL.) 17 November 1992, column 3, lines 64-66	1-41
Y	US, A, 5,252,216 (FOLENA-WASSERMAN ET AL.) 12 October 1993, column 2, lines 53-59	1-41

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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23 MARCH 1995	11APR1995
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